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THE T CELL ANTIGEN RECEPTOR COMPLEX EXPRESSED ON NORMAL PERIPHERAL BLOOD CD4$^-$,CD8$^-$ T LYMPHOCYTES

A CD3-associated Disulfide-linked γ Chain Heterodimer

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The T cell antigen receptor (TCR)[1] expressed on most T lymphocytes is an ~90 kD disulfide-linked heterodimer, composed of α and β glycoprotein subunits. The α/β TCR heterodimer is noncovalently associated with CD3, a glycoprotein complex containing three or more invariant proteins (1–6, reviewed in references 7, 8). The TCR is responsible for specific antigen recognition; whereas the invariant CD3 complex has been implicated in signal transduction (7, 8). Like immunoglobulin genes, the genes encoding TCR-α and -β chains are composed of variable (V), joining (J), and constant (C) genetic elements that undergo rearrangement during T cell differentiation (9–12; reviewed in reference 13). Several mAbs have been generated against the invariant CD3 proteins and against idiotypic determinants of the TCR. One mAb, WT31, reacts with a framework determinant present on all α/β TCR heterodimers (14, 15). Using mAbs or probes for the TCR-α and -β gene products, it has been established that most mature T lymphocytes in vivo and most in vitro–cultured T cell lines express antigen receptors using the α/β heterodimer (reviewed in references 7, 8, 13).

In addition to α and β, a third gene, γ, has been shown to rearrange in T lymphocytes (16–21). γ genes are also composed of V, J, and C genetic elements (16, 19, 21). γ genes are rearranged and transcribed before β and α genes during T cell ontogeny and differentiation (22–24). Recently, T lymphocytes expressing γ mRNA, but not α mRNA, have been identified (25–27). PEER, a T leukemia cell line, expresses a non–disulfide-linked ~55–60 kD glycoprotein that is noncovalently associated with the CD3 complex on the plasma membrane (25). Lack

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[1] *Abbreviations used in this paper:* Chaps, 3(3-cholamidopropyl-dimethylammonio)-1-propane sulfonate; NEPHGE, nonequilibrium pH gradient electrophoresis; PE, phycoerythrin; PHA-M, phytohemagglutinin-M; TCR, T cell antigen receptor.

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of α mRNA, the presence of apparently functional γ mRNA, and reactivity of the CD3-associated ~55–60 kD glycoprotein with an anti-γ peptide antiserum clearly indicate that the antigen receptor complex on PEER uses γ, rather than α or β gene products (28). IL-2-dependent T cell lines established from patients with immunodeficiency diseases (26) and $CD4^-$,$CD8^-$ thymocytes (27) also express CD3-associated, non–disulfide-linked glycoproteins of ~55 and ~40 kD on the cell surface. Like the PEER cell line, these IL-2-dependent cell lines transcribed γ mRNA, but not α mRNA, and antiserum against γ peptides reacted with the CD3-associated ~55 kD structure (26, 27). All of these cell lines reacted with antibodies against CD3, but did not react with WT31, an mAb directed against the α/β TCR heterodimer (25–27).

The existence of $CD3^+$, $WT31^-$ (TCR-α/β^-) cell lines prompted us to examine whether T lymphocytes with this phenotype were present in peripheral blood and thymus obtained from normal individuals. $CD3^+$,$WT31^-$ T lymphocytes were present at low frequency in peripheral blood (~3% of PBL/T cells) and thymus (~0.5% of thymocytes) (29). Surprisingly, essentially all $CD3^+$,$WT31^-$ PBL/T cells and thymocytes expressed neither the CD4 nor CD8 T cell differentiation antigens (29). In contrast, all $CD3^+$,$WT31^+$ T lymphocytes and thymocytes expressed CD4 and/or CD8 (29). In previous studies (30), we established IL-2-dependent T cell lines from $CD3^+$,4^-,8^- PBL/T lymphocytes which stably maintained this phenotype in culture. These IL-2-dependent $CD3^+$,4^-,8^- T cell lines are functionally competent and mediate non–MHC-restricted cytotoxicity against a variety of NK-sensitive and NK-insensitive tumor cell targets (30). In the present study, we demonstrate that these $CD3^+$,4^-,8^- T cell lines established from the peripheral blood of normal, healthy adult donors do not express α/β TCR, but instead express disulfide-linked antigen receptors using γ gene–encoded proteins.

Materials and Methods

Monoclonal Antibodies. Leu series mAbs were produced by the Becton Dickinson Monoclonal Center, Inc. (Mountain View, CA). The CD3, CD4, CD5, and CD8 differentiation antigens were detected by using anti-Leu-4, anti-Leu-3, anti-Leu-1, and anti-Leu-2 mAbs, respectively (31). The WT31 mAb, directed against the TCR-α/β heterodimer, was generously provided by Dr. Wil Tax, University of Nijmegen, The Netherlands (14, 15). An mAb against a framework determinant of the TCR β chain (βF1) was generously provided by Dr. Mike Brenner, Dana-Farber Cancer Institute, Boston, MA (26).

Immunofluorescence and Flow Cytometry. Methods of immunofluorescence, flow cytometry, and data analysis have been described previously (32). Fluorochrome-conjugated, isotype-matched Ig that do not react with human leukocytes were used to control for nonspecific binding in the immunofluorescence assays. Fluorescence was measured by using a FACS 440 or FACStar flow cytometer and data were analyzed by using a Consort 30 or Consort 40 Data Analysis System (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Isolation and Culture of Peripheral Blood $CD3^+$,4^-,8^- T Lymphocytes. Mononuclear cells from normal peripheral blood (Stanford Blood Center, Stanford, CA) were isolated using Ficoll/Hypaque. Monocytes were depleted by plastic adherence. Nonadherent cells were stained with a mixture of FITC-conjugated anti-Leu-2 (CD8), FITC-conjugated anti-Leu-3 (CD4), and phycoerythrin (PE)-conjugated anti-Leu-4 (CD3). After extensive washing in ice-cold PBS (0.01 M phosphate, pH 7.2), the cells were resuspended in PBS containing 1% FCS. Lymphocytes expressing CD3, but lacking both CD4 and CD8, therefore demonstrated specific red (PE) fluorescence, but not green (FITC) fluorescence.

$CD3^+,4^-,8^-$ cells were isolated to >95% purity by cell sorting using a FACS. Cells were cultured in RPMI 1640 supplemented with 10% FCS (KC Biologicals, Lexena, KS), 1 mM glutamine, 100 μg/ml gentamicin, and 1,000 U/ml rIL-2 (Cetus Corp., Emeryville, CA).

Mitogenic Stimulation. PBMC were cultured at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS, 1 mM glutamine, 100 μg/ml gentamicin, and 2 μg/ml PHA-M (Gibco Laboratories, Grand Island, NY). Cells were harvested after 3 d and washed extensively in PBS.

Cell Lines. The HPB-ALL T leukemia cell line was generously provided by Dr. J. Minowada (Fujisaki Cell Center, Okayama, Japan), the HUT 78 T leukemia cell line was obtained from the American Type Culture Collection (Rockville, MD), and the DAUDI B cell line was obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). The cloned, IL-2-dependent NK cell line, NK1A3, was generously provided by Drs. B. Gemlo and A. Rayner, University of California, Los Angeles, CA.

Immunoprecipitation and SDS-PAGE Analysis. Cells were labeled with ^{125}I (Amersham Corp., Arlington Heights, IL) by using the glucose oxidase/lactoperoxidase method (33). $1-5 \times 10^6$ ^{125}I-labeled cells were washed three times in 1 ml PBS containing 10 mM iodoacetamide, and were lysed in 0.5 ml lysis buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.02% NaN_3, pH 8.0) containing 20 KI units/ml aprotinin (Sigma Chemical Co., St. Louis, MO), 10 mM iodoacetamide, and 5 mM Chaps (3[3-cholamidopropyl-dimethylammonio]-1-propane sulfonate) (Calbiochem-Behring Corp., San Diego, CA)) for 45 min at 4°C. Cells were pelleted by centrifugation at 13,000 *g* for 5 min. For removal of unbound ^{125}I, the lysates were passed through a 0.2 ml (bed volume) column of Dowex 1X8-400 (chloride form; Sigma Chemical Co.), equilibrated in lysis buffer. After this procedure, >85% of the radioisotope present in the lysate was precipitated by 10% TCA. Before specific immunoprecipitation, lysates were precleared twice by incubation with 10 mg of packed formalin-fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring Corp.) coated with rabbit anti–mouse IgG (Pel-Freeze Biologicals, Rogers, AR). For specific immunoprecipitation, 50 μl *S. aureus* (Pansorbin, 10% wt/vol solution) were incubated for 15 min at 4°C with saturating amounts of rabbit anti–mouse IgG antiserum, washed twice in lysis buffer by centrifugation at 13,000 *g* for 15 s, then incubated for 1–2 h with 1–5 μg of specific mAb in a total volume of 100 μl of lysis buffer. mAb-coated *S. aureus* were washed once in lysis buffer, then 50–100 μl of ^{125}I-labeled cell lysate was added to the mAb-coated *S. aureus* pellet. After 2 h of incubation at 4°C, the *S. aureus* were washed five times in lysis buffer containing 0.5 M NaCl. Bound material was eluted by boiling in sample buffer containing 2.3% SDS with or without 5% 2-ME (34). Samples were analyzed by SDS-PAGE using a discontinuous buffer system (34). Two-dimensional gel electrophoresis, using nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and SDS-PAGE using 10% acrylamide gels in the second dimension, was performed as described by Jones (35). pH 3.5–10 ampholines (LKB Instruments Inc., Gaithersburg, MD) were used in NEPHGE analysis. Two-dimensional diagonal gel electrophoresis, using SDS-PAGE under nonreducing conditions in the first dimension, followed by SDS-PAGE under reducing conditions in the second dimension, was performed as described (1). Protein standards (reducing: Sigma Chemical Co.; nonreducing, Pharmacia Fine Chemicals, Piscataway, NJ) were included for molecular weight determination. Gels were dryed and autoradiographed at −70°C on Kodak XR film using intensifying screens (Cronex Lightening Plus; DuPont Co., Wilmington, DE).

Anti-γ Antiserum. A peptide corresponding to the predicted amino acid sequence of Cγ1 derived from nucleotides 237–281 was synthesized using a solid-phase peptide synthesizer (Peninsula Laboratories, Inc., Belmont, CA) (36). The peptide was purified by HPLC and coupled to KLH at a ratio of 5 mg:1 mg (carrier/peptide). Rabbits were immunized subcutaneously with 100 μg of specific peptide conjugate emulsified in CFA and boosted twice at 3-wk intervals before bleeding. Specific reactivity of the antipeptide antiserum was demonstrated in an enzyme-linked immunoassay.

Immunoprecipitation of γ Chain. The CD3/TCR complex was immunoprecipitated from Chaps detergent lysates of ^{125}I-labeled cells using formalin-fixed *S. aureus* coated with anti-Leu-4 mAb (as described above). The CD3/TCR complex was eluted from the

immunoabsorbent by incubation for 30 min at 56°C in 100 μl of lysis buffer containing 1% SDS and 2 mM DTT (26). Samples were alkylated and partially renatured by dilution in four volumes of Tris-buffered saline (0.05 M Tris, 0.15 M NaCl, pH 7.4) containing 1.5% NP-40 detergent and 20 mM iodoacetamide. Samples were subsequently divided into aliquots and incubated with 50 μl formalin-fixed *S. aureus* (Pansorbin; 10% vol/vol solution) precoated with either 10 μl rabbit anti-Cγ peptide serum or normal rabbit serum. After incubation for 2 h at 4°C, the immunoabsorbent was washed in lysis buffer. Bound material was eluted in sample buffer containing 2.3% SDS and 5% 2-ME. Samples were analyzed by SDS-PAGE using 10% acrylamide gels.

Probes. A murine β cDNA probe ($TM86_{T5}$) (37) and a murine Cα probe (38) were generously provided by Dr. M. Davis (Stanford University, Stanford, CA) and Dr. L. Hood (Cal Tech, Pasadena, CA), respectively. A human genomic Jγ probe (pH60) (18) was generously provided by Dr. T. Rabbitts (Medical Research Council, Cambridge, United Kingdom). A murine γ cDNA probe (8A2) was isolated from a cDNA library constructed from a C57BL/6 murine T leukemia cell line, C6VL (1), and was subcloned into pUC8. A 0.6-kb Pvu II/Ava I insert, corresponding to a J-C region segment of the γ gene (nucleotides 444–1,028 in the γ cDNA sequence [16]) was isolated and was used as probe for Northern blot analysis.

Southern and Northern Blot Analysis. Cells were washed with PBS and pelleted in a microfuge tube. Cytoplasmic RNA was isolated as described by Favaloro et al. (39), with minor modifications. Nuclei were pelleted and used for subsequent genomic DNA purification according to the method of Law et al. (40), with the addition of 40 μl 5% Sarkosyl/0.5 M EDTA to the usual lysis buffer to ensure complete lysis. Genomic DNA was digested with restriction enzymes (New England Biolabs, Beverly, MA) and was electrophoresed in 0.8% agarose gels (41). After electrophoresis, the gels were stained with ethidium bromide to verify the integrity of the DNA and to confirm that comparable amounts of DNA were present in all lanes. RNA was electrophoresed in 1.0% agarose gels (41). RNA and DNA were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA) and baked for 2 h in a vacuum oven at 80°C (41). Probe inserts were isolated and were labeled with [^{32}P]dCTP (Amersham Corp.) (>10^9 cpm/μg DNA) by the method of Feinberg and Vogelstein (42). Membranes were hybridized at 65°C for 24 h with ~2 × 10^7 cpm of [^{32}P]dCTP-labeled probe in 35 ml hybridization buffer (5× SSPE [1× SSPE: 0.15 NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1mM EDTA, pH 7.4], 1× Denhardt's, 0.3% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA). After hybridization, membranes were washed twice for 15 min and twice for 30 min (room temperature) in 10 mM sodium phosphate, 1 mM disodium EDTA, and 0.2% SDS. After a final wash for 45 min at 65°C, the membranes were autoradiographed. Fragment sizes were determined based on standards purchased from Bethesda Research Laboratories (Bethesda, MD).

Results and Discussion

IL-2-dependent $CD3^+,4^-,8^-$ Cell Lines Lack Reactivity with WT31 mAb. ~3% of normal peripheral blood $CD3^+$ T lymphocytes express neither CD4 nor CD8 differentiation antigens (30). $CD3^+,4^-,8^-$ peripheral blood T lymphocytes were isolated to >95% purity and were established in culture using rIL-2 as growth factor (30). $CD3^+,4^-,8^-$ T cell lines have been established from >15 normal donors, and >90% of lymphocytes stably maintained the phenotype, $CD3^+,4^-,8^-$. To characterize the TCR, IL-2-dependent $CD3^+,4^-,8^-$ cell lines derived from 15 independent donors were stained with WT31, an mAb directed against the TCR-α/β heterodimer. None of these $CD3^+,4^-,8^-$ cell lines reacted with WT31, although all cell lines reacted with anti-Leu-4, an mAb directed against CD3. Data from a representative $CD3^+,4^-,8^-$ cell line is presented in Fig. 1. As noted previously (30), a small fraction of cells within these IL-2-dependent $CD3^+,4^-,8^-$ cell lines expressed very low levels of CD8 antigen (approximately one-tenth the

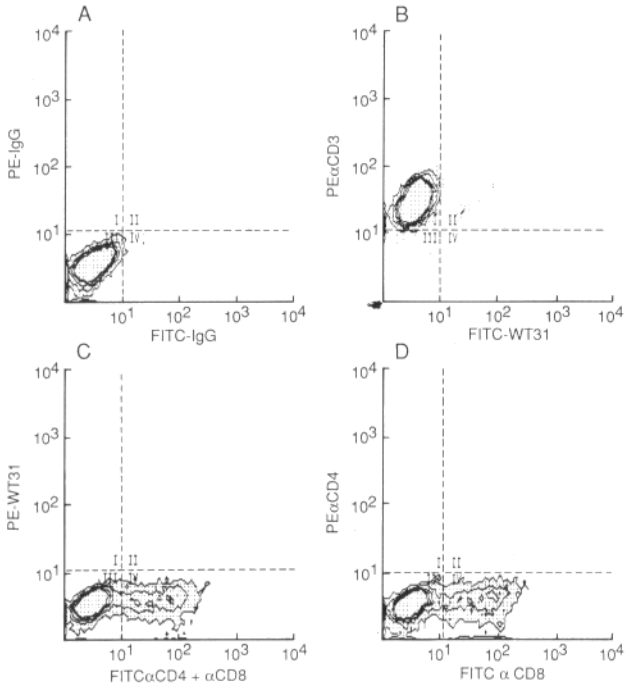

FIGURE 1. IL-2-dependent peripheral blood $CD3^+,4^-,8^-$ T cell lines lack reactivity with antibody WT31 (anti-TCR-α/β). IL-2-dependent $CD3^+,4^-,8^-$ peripheral blood T cell lines were stained with: (*A*) FITC-conjugated IgG and PE-conjugated IgG control mAb; (*B*) FITC-conjugated WT31 and PE-conjugated anti-Leu-4 (CD3); (*C*) FITC-conjugated anti-Leu-2 (CD8) plus FITC-conjugated anti-Leu-3 (CD4) and PE-conjugated WT31; and (*D*) FITC-conjugated anti-Leu-2 (CD8) and PE-conjugated anti-Leu-3 (CD4). Samples were analyzed by flow cytometry. Correlated green (FITC: abscissa, four decade scale) and red (PE: ordinate, four decade scale) fluorescence were displayed as contour plots. Based on the control sample, contour plots were divided into quadrants to represent unstained cells (*lower left*), cells stained only with PE (*upper left*), cells stained only with FITC (*lower right*), and cells stained with both FITC and PE (*upper right*).

amount of CD8 antigen expressed on most peripheral blood T lymphocytes). However, as shown in Fig. 1, even these dim $CD8^+$ cells failed to react with WT31. We suspect that these dim $CD8^+$ lymphocytes were generated from the $CD3^+,4^-,8^-$ population as a consequence of in vitro culture; however, we cannot exclude the possibility that these cells were a contaminant in the isolation of peripheral blood $CD3^+,4^-,8^-$ lymphocytes. $CD4^+$ cells were never detected in these cell lines. These results are consistent with our recent studies (29) demonstrating that WT31 mAb fails to react with the ~3% of normal peripheral blood $CD3^+$ T lymphocytes that coexpress neither CD4 nor CD8. Hence, these cultured cell lines appear to be derived from and representative of the $CD3^+,4^-,8^-$ normal PBL/T cell subset.

Biochemical Characterization of the CD3-associated Proteins Expressed on $CD3^+,4^-,8^-$ ($WT31^-$) T Cell Lines. Prior studies have demonstrated that the TCR-α/β heterodimer is noncovalently associated with the CD3 glycoprotein complex (1–8). Using certain detergent systems, e.g., Chaps (25) and digitonin (43), it has been possible to preserve this association and coimmunoprecipitate the intact CD3/TCR complex using antibodies either against CD3 or TCR. This technique was used to identify the CD3-associated structures expressed on IL-2-dependent $CD3^+,4^-,8^-$ cell lines. $CD3^+,4^-,8^-$ cells (>95% $WT31^-$) were radiolabeled with ^{125}I, solubilized in Chaps detergent lysis buffer, and immunoprecipitated with anti-Leu-4 (CD3) mAb. Immune complexes were analyzed by SDS-PAGE. IL-2-dependent $CD3^+,4^-,8^-$ ($WT31^-$) cell lines established from five independent normal individuals were assayed. Representative data from two cell lines are shown in Fig. 2. A disulfide-linked structure of ~90 kD was coimmunoprecipi-

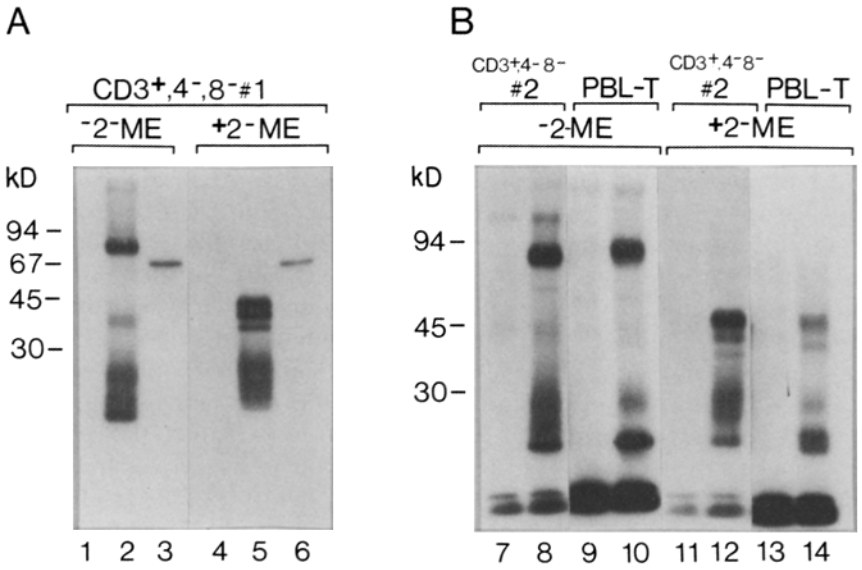

FIGURE 2. Immunoprecipitation and SDS-PAGE analysis of the CD3/TCR complex expressed on $CD3^+,4^-,8^-$ T cell lines. IL-2-dependent $CD3^+,4^-,8^-$ cell lines from two different donors (No. 1, lanes *1–6*; and No. 2, lanes *7, 8, 11, 12*) and normal peripheral blood T lymphocytes (*PBL-T*; lanes *9, 10, 13, 14*) were radiolabeled with ^{125}I and were detergent solubilized. Both $CD3^+,4^-,8^-$ cell lines were >95% $WT31^-$; whereas normal PBL-T cells were ~97% $WT31^+$, as determined by flow cytometry. Lysates were immunoprecipitated using *S. aureus* coated with control Ig (lanes *1, 4, 7, 11,* and *13*), anti-Leu-4 (CD3) (lanes *2, 5, 8, 10, 12,* and *14*), or anti-Leu-1 (CD5) (lanes *3* and *6*). Immune complexes were eluted from *S. aureus* in sample buffer containing 2.3% SDS, either with or without 5% 2-ME. Immunoprecipitates were analyzed using 12.5% (*A*) or 10% (*B*) acrylamide gels. Note that the ~38-kD band seen in lane *2* has not been consistently observed and may be the result of partial sample reduction in this experiment.

tated with the CD3 complex (22–29 kD). Under reducing conditions, the 90-kD structure dissociated into three subunits of ~45, ~40, and ~37 kD. The CD3-associated ~90 kD heterodimer expressed on two of the five $CD3^+,4^-,8^-$ ($WT31^-$) cell lines examined dissociated into only two subunits of ~45 and ~40 kD upon reduction (not shown). These structures were not coimmunoprecipitated with anti-Leu-1 (CD5) or control mAb (Fig. 2*A*). Furthermore, these CD3-associated structures were not coimmunoprecipitated with anti-Leu-4 in detergent systems (e.g., NP-40) that dissociated the CD3/TCR complex (not shown). Immunoprecipitation of the CD3/TCR-α/β heterodimer complex from normal PBL/T cells (~97% $WT31^+$) is shown for comparison (Fig. 2*B*). Normal PBL/T lymphocytes demonstrated an ~95 kD heterodimer (nonreduced) that dissociated into diffuse bands of ~47 and ~40 kD upon reduction.

Two-dimensional "diagonal" gel electrophoresis was used to determine the relationship of the ~45, ~40, and ~37 kD (reduced) subunits to the single ~90 kD (nonreduced) structure. Anti-Leu-4 (CD3) immunoprecipitates were separated by SDS-PAGE under nonreducing conditions in the first dimension, followed by reduction and separation by SDS-PAGE under reducing conditions in the second dimension. Using this technique, structures with interchain disulfide bonds were dissociated in the second dimension, and thus were observed below the diagonal; whereas, non–disulfide-linked structures were observed on

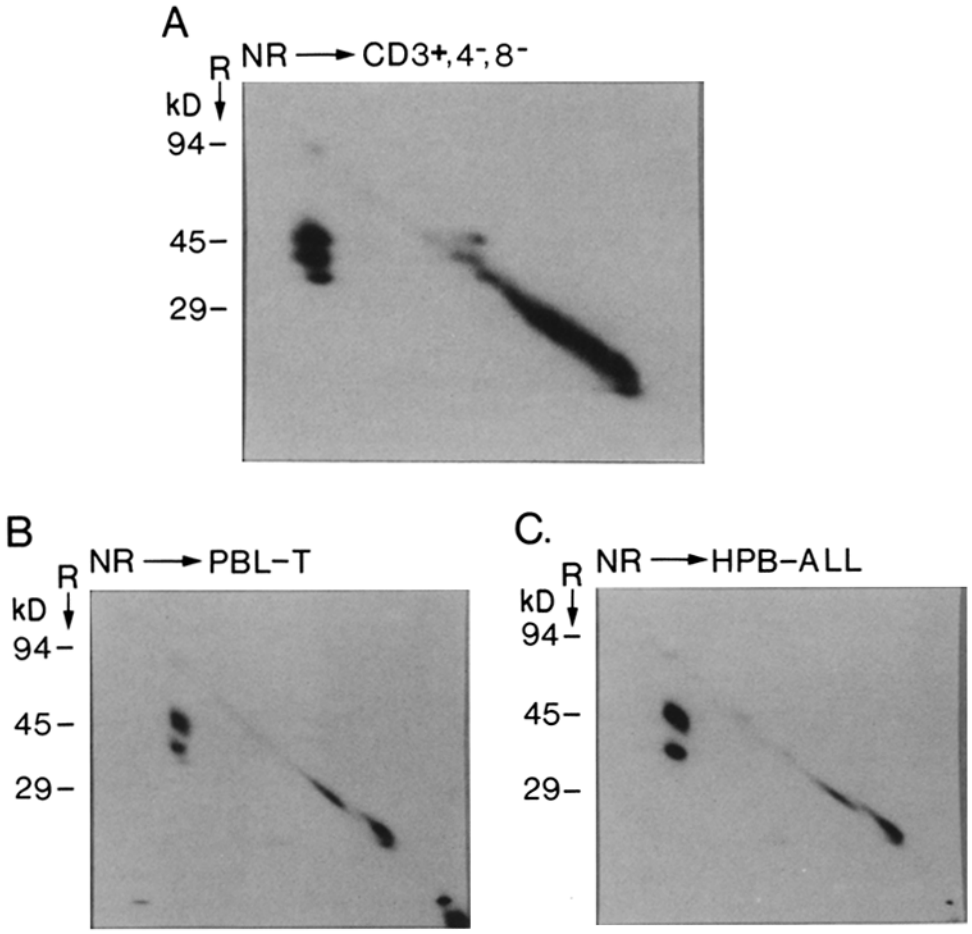

FIGURE 3. Diagonal gel electrophoresis analysis of the CD3/TCR complex expressed on $CD3^+,4^-,8^-$ T cells. Detergent-solubilized lysates of ^{125}I-labeled IL-2-dependent $CD3^+,4^-,8^-$ ($WT31^-$) cells (same sample as lane *2* in Fig. 2) (*A*), normal PBL-T cells (*B*), and the HPB-ALL tumor cells (*C*) were immunoprecipitated with anti-Leu-4 (CD3). Samples were analyzed by two-dimensional gel electrophoresis (1st dimension, SDS-PAGE nonreducing; 2nd dimension, SDS-PAGE reducing) using 10% acrylamide gels. As noted in Fig. 2, the minor band above the diagonal probably represents heterodimer that was partially reduced during preparation, since the mobility of this protein is essentially identical to the ~45 kD subunit below the diagonal.

the diagonal. As shown in Fig. 3*A*, all three ~45, ~40, and ~37 kD subunits were predominately observed directly below the diagonal, indicating that the ~90 kD (nonreduced) structure is composed primarily of disulfide-linked dimeric pairs of these subunits. Careful examination of the relative mobility of these subunits suggested that these proteins may form dimers composed of ~45/~40 kD and ~45/~37 kD; however, further studies will be necessary to confirm this notion. Since the $CD3^+,4^-,8^-$ cell lines used in the present experiments were not cloned, this observation may reflect heterogeneity within the cell population. Thus, we cannot exclude the alternative possibility that these subunits assemble as homodimers, rather than heterodimers. Diagonal gel electrophoresis of the CD3/TCR complex immunoprecipitated from HPB-ALL tumor cells and normal

PBL/T lymphocytes are shown for comparison (Fig. 3, *B* and *C*). Prior studies have established that HPB-ALL cells express a CD3-associated heterodimer composed of α and β subunits that reacts with the WT31 mAb (15, 44).

The CD3/TCR complex expressed on $CD3^+,4^-,8^-$ cells was further analyzed by two-dimensional electrophoresis using pH-dependent separation in the first dimension, followed by size separation in the second dimension. NEPHGE analysis revealed that the ~37 and ~40 kD subunits migrated with essentially identical mobility, whereas the ~45 kD structure was substantially more basic (Fig. 4). It is possible that the ~37 and ~40 kD subunits are differentially glycosylated species of the same protein. The ~45 kD subunit appears to be a distinct polypeptide, unrelated to the ~37 and ~40 kD subunits. Further studies support this notion (see below). It should be noted that no α-like acidic subunits were observed in the CD3/TCR complex isolated from $CD3^+,4^-,8^-$ cells. In contrast, NEPHGE analysis of the CD3/TCR structure expressed on HPB-ALL demonstrated a relatively acidic subunit of ~45 kD (the α chain) and a relatively basic subunit of ~37 kD (the β chain).

Reactivity of the CD3-associated Heterodimer with Anti-γ Antiserum, But not Anti-β Framework mAbs. Detergent (Chaps) lysates of ^{125}I-labeled $CD3^+,4^-,8^-$ cell lines and HPB-ALL tumor cells were incubated with *S. aureus* coated with βF1 mAb. Brenner et al. (26) have previously demonstrated that βF1 mAb specifically reacts with a framework determinant on the TCR β chain. Furthermore, it has been shown that anti-βF1 mAb reacts with >95% of normal PBL-T lymphocytes, by immunofluorescent cytoplasmic staining techniques (26). As shown in Fig. 5, anti-Leu-4 (CD3) mAb immunoprecipitated a CD3-associated heterodimer from both HPB-ALL cells and the $CD3^+,4^-,8^-$ cell line. However, whereas βF1 clearly reacted with the heterodimer present on HPB-ALL tumor cells, this antibody did not react with the CD3-associated heterodimer expressed on the $CD3^+,4^-,8^-$ cell line.

The CD3-associated heterodimer expressed on $CD3^+,4^-,8^-$ cells did react with an antiserum directed against a synthetic Cγ-chain peptide. The CD3/TCR heterodimer immunoprecipitated from $CD3^+,4^-,8^-$ cells was eluted from anti-Leu-4 (CD3) immunoadsorbent. After denaturation and reduction, samples were renatured and alkylated. These proteins were reprecipitated with anti-Cγ peptide serum, or normal rabbit serum. As shown in Fig. 6, anti-Cγ peptide serum specifically reacted with the ~37 and ~40 kD CD3-associated subunits, but not with the CD3 polypeptides or the ~45 kD subunit. Anti-Cγ peptide serum failed to react with either the α chain or β chain of the heterodimer expressed on HPB-ALL or JURKAT tumor cells (not shown). These results suggest that the ~37 and ~40 kD subunits expressed on $CD3^+,4^-,8^-$ cells are γ chain–encoded proteins. The nature of the higher molecular mass ~45-kD subunit is unknown. However, the possibility that this polypeptide is a β chain product was excluded by the observation that βF1 mAb failed to react with this structure.

Transcription of TCR Genes in $CD3^+,4^-,8^-$ ($WT31^-$) Cell Lines. To further characterize these $CD3^+,4^-,8^-$ ($WT31^-$) cell lines, RNA was isolated and analyzed by Northern blot analysis for the presence of α, β, and γ transcripts. As shown in Fig. 7, $CD3^+,4^-,8^-$ ($WT31^-$) cells contained abundant γ gene transcripts of ~1.6 kb. In contrast, we failed to detect a significant amount of full-length 1.3-kD β mRNA, although small amounts of 1.0-kb β (presumably nonfunctional)

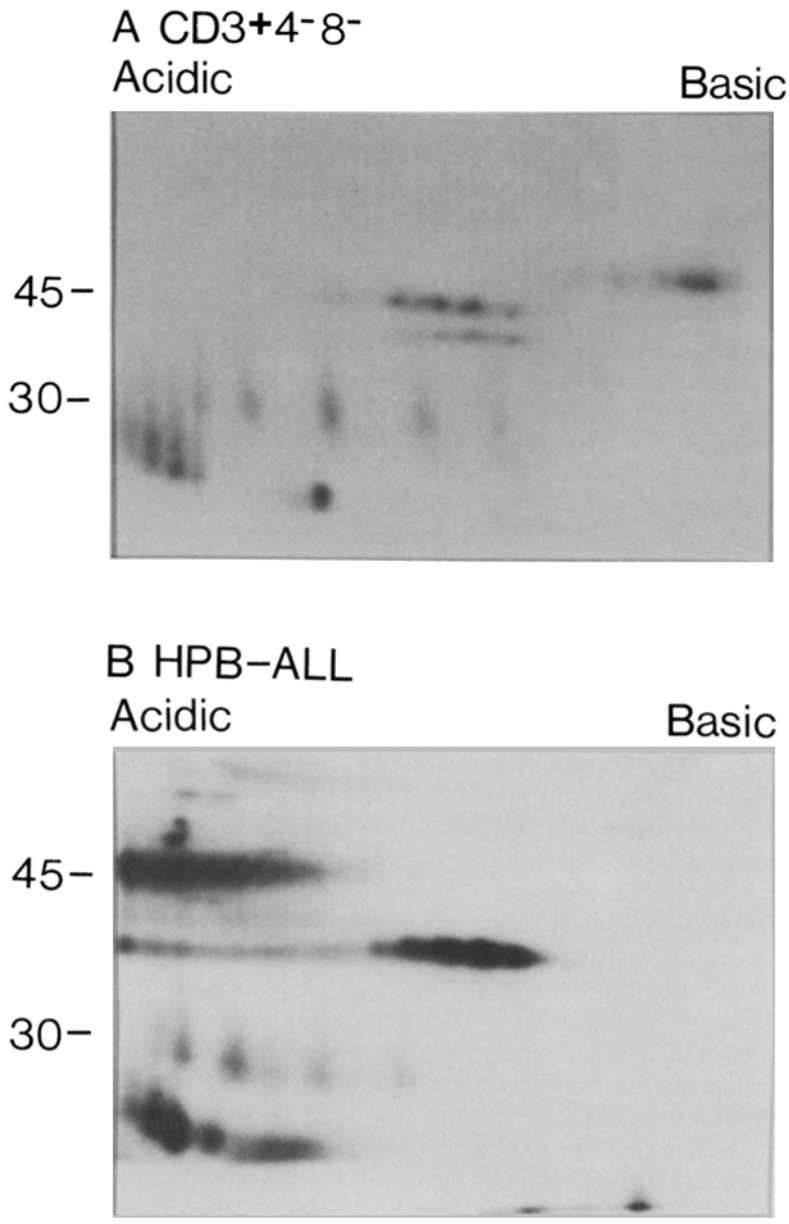

FIGURE 4. NEPHGE analysis of the CD3/TCR complex expressed on $CD3^+,4^-,8^-$ T cells. Detergent solubilized lysates of ^{125}I-labeled IL-2-dependent $CD3^+,4^-,8^-$ ($WT31^-$) cells (*A*) and the HPB-ALL tumor cells (*B*) cells were immunoprecipitated with anti-Leu-4 (CD3). Samples were analyzed by NEPHGE. 10% acrylamide gels were used for SDS-PAGE analysis in the 2nd dimension.

mRNA were detected. Similarly, only very low amounts of 1.6-kb α mRNA were detected in the $CD3^+,4^-,8^-$ cell line. We cannot exclude the possibility that this small amount of α mRNA was transcribed by a minor population of contaminating $CD3^+,WT31^+$ T lymphocytes (composing 2–5% of these cell lines). A 1.1-kb α transcript was also observed; the significance of this short α transcript is

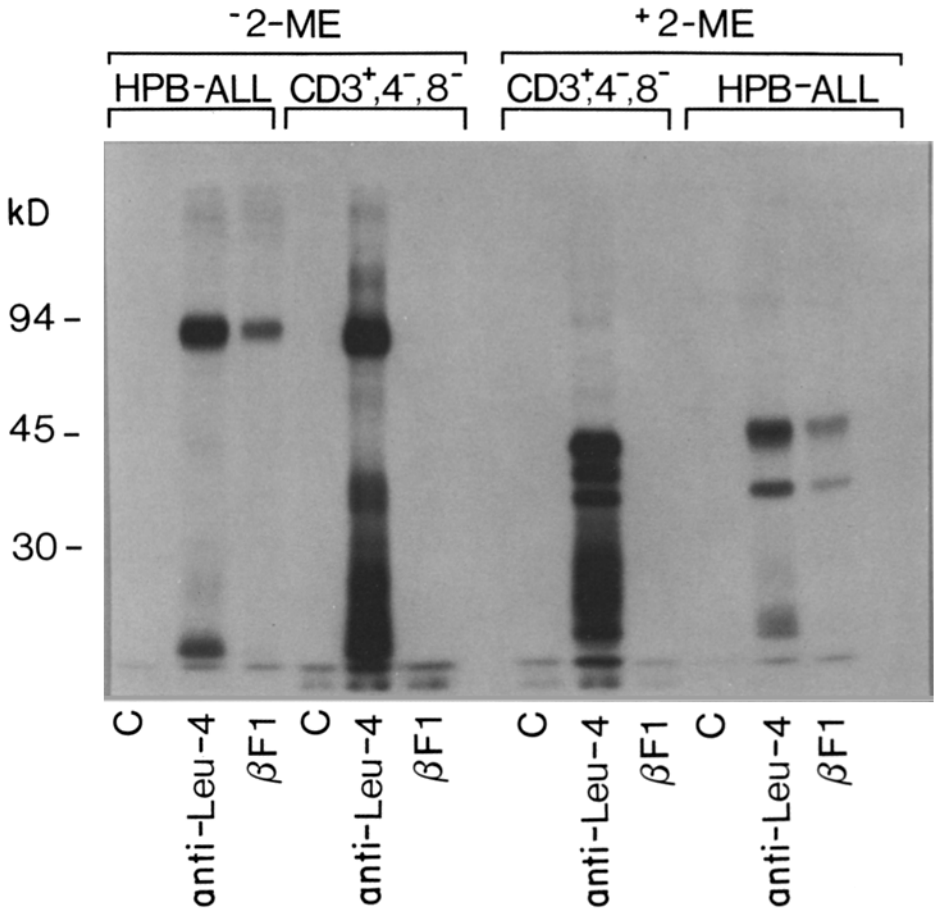

FIGURE 5. Immunoprecipitation with anti-β chain mAb. Detergent solubilized lysates of ^{125}I-labeled IL-2-dependent CD3$^+$,4$^-$,8$^-$ (WT31$^-$) cells and HPB-ALL tumor cells were immunoprecipitated with: control Ig (*C*), βF1, and anti-Leu-4 (CD3), as indicated. Immune complexes were eluted from *S. aureus* in sample buffer containing 2.3% SDS, either with or without 5% 2-ME. Samples were analyzed by SDS-PAGE using 10% acrylamide gels.

unknown. As controls, mRNA from PHA-activated peripheral blood T lymphoblasts from normal donors and HPB-ALL T leukemia tumor cells were analyzed. PHA T lymphoblasts and HPB-ALL cells that reacted with WT31 mAb expressed apparently competent 1.3-kb β and 1.6-kb α mRNA. γ mRNA was not detected in PHA-activated T lymphoblasts. Although γ mRNA was detected in HPB-ALL tumor cells, there is no evidence that this transcript encodes functional protein.

Rearrangement of TCR Genes in CD3$^+$,4$^-$,8$^-$ (WT31$^-$) Cell Lines. In humans, two Cγ genes, each with associated J elements, have been identified (18, 19). Rearrangement of γ genes is readily detected by Southern blot analysis using J region probes (18, 19). Genomic DNA from CD3$^+$,4$^-$,8$^-$ (WT31$^-$) cells was isolated, digested with Eco RI restriction enzyme, and assayed by Southern blot analysis using a Jγ probe. DNA isolated from cloned T leukemia cell lines (HPB-ALL and HUT 78) contained rearranged fragments consistent with monoclonal cell populations. Only unrearranged, germline fragments were detected in a B

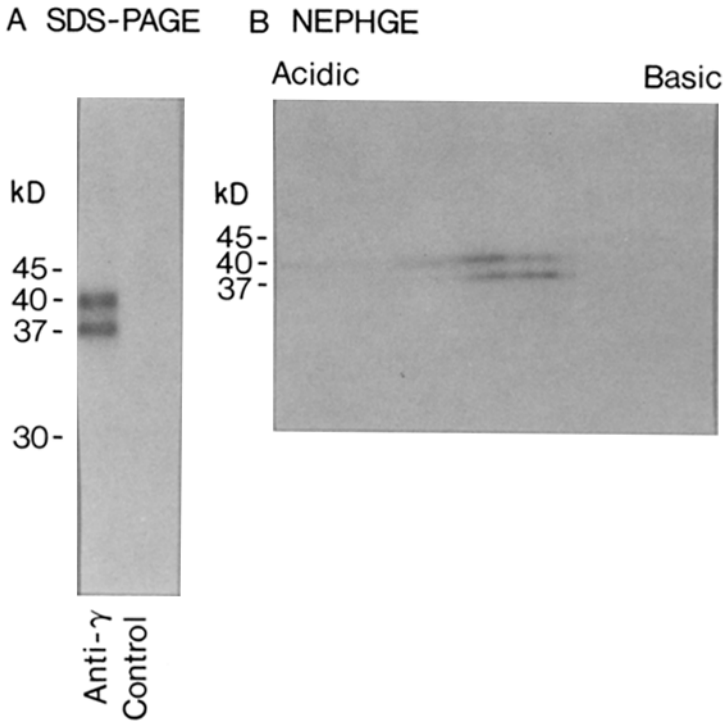

FIGURE 6. Immunoprecipitation with anti-γ antiserum. Detergent-solubilized lysates of ^{125}I-labeled IL-2-dependent CD3⁺,4⁻,8⁻ (WT31⁻) cells (same cell line as used in Fig. 5) were immunoprecipitated with anti-Leu-4 (CD3). Immune complexes were eluted from *S. aureus* and reimmunoprecipitated using rabbit anti-γ peptide serum or normal rabbit serum (control) as described in Materials and Methods. Samples were analyzed by SDS-PAGE (*A*) and NEPHGE (*B*) using 10% acrylamide gels.

cell line (DAUDI). γ genes were rearranged in both CD3⁺,4⁻,8⁻ cells and normal PBL/T (~97% WT31⁺) lymphocytes (Fig. 8).[2] The rearranged fragments seen in the CD3⁺,4⁻,8⁻ (WT31⁻) cell line were similar to the rearranged fragments seen in normal polyclonal PBL/T lymphocytes isolated from two independent donors. These results suggest that the CD3⁺,4⁻,8⁻ cell line is polyclonal, rather than monoclonal. Similar results were also obtained using CD3⁺,4⁻,8⁻ (WT31⁻) cell lines established from two different donors (not shown). DNA from CD3⁺,4⁻,8⁻ (WT31⁻) cell lines were also digested with Kpn I, another restriction enzyme that allows detection of γ gene rearrangement in polyclonal PBL/T cells (45). Polyclonal rearrangement of γ genes in the CD3⁺,4⁻,8⁻ (WT31⁻) cell line was similarly observed (not shown).

The genomic organization of β genes in CD3⁺,4⁻,8⁻ (WT31⁻) cell lines was also examined and compared with polyclonal PBL/T lymphocytes (Fig. 9). DNA from a B cell line (DAUDI) and a cloned, CD3⁻ NK cell line were not rearranged, and served as germline controls in these experiments. Using Eco RI restriction enzyme analysis, ~10 and ~3.7 kb fragments representing germline Cβ1 and Cβ2 genes, respectively, were detected (46). In germline DNA, the 10- and 3.7-

[2] Although the intensity of rearranged γ gene fragments varied between different blood donors, identical fragments were observed in all PBL/T cell populations examined (45). No polymorphism has been detected using Eco RI restriction enzyme analysis (45).

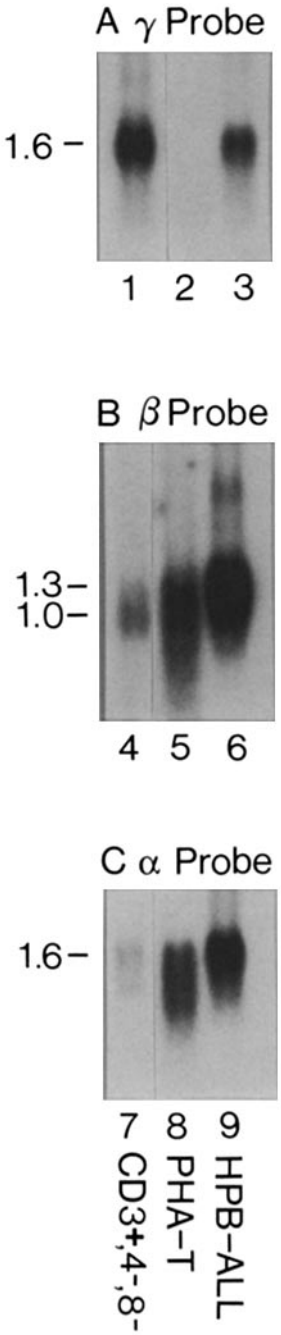

FIGURE 7. Northern blot analysis of TCR gene products expressed by $CD3^+,4^-,8^-$ T cells. RNA were isolated from an IL-2-dependent $CD3^+,4^-,8^-$ ($WT31^-$) cell line (lanes *1*, *4*, and *7*), PHA-activated T lymphocytes from normal donors (lanes *2*, *5*, and *8*) and HPB-ALL T leukemia cells (lanes *3*, *6*, and *9*). Equal amounts of each RNA (12 μg/lane) were analyzed by the Northern blot technique, using ^{32}P-labeled γ (*A*), β (*B*), and α (*C*) probes. Membranes B and C were rehybridized with γ probe to ensure that lanes *1*, *4*, and *7* contained equivalent amounts of RNA (not shown).

kb bands were approximately equal in intensity; whereas, rearrangement of Cβ1 genes resulted in a reduction of the 10-kb band (46). Consistent with prior reports (46, 47), normal, polyclonal PBL/T lymphocytes demonstrated extensive rearrangement of Cβ1 genes, resulting in significant loss of the germline 10-kb fragment. Like PBL/T lymphocytes, $CD3^+,4^-,8^-$ ($WT31^-$) cell lines demon-

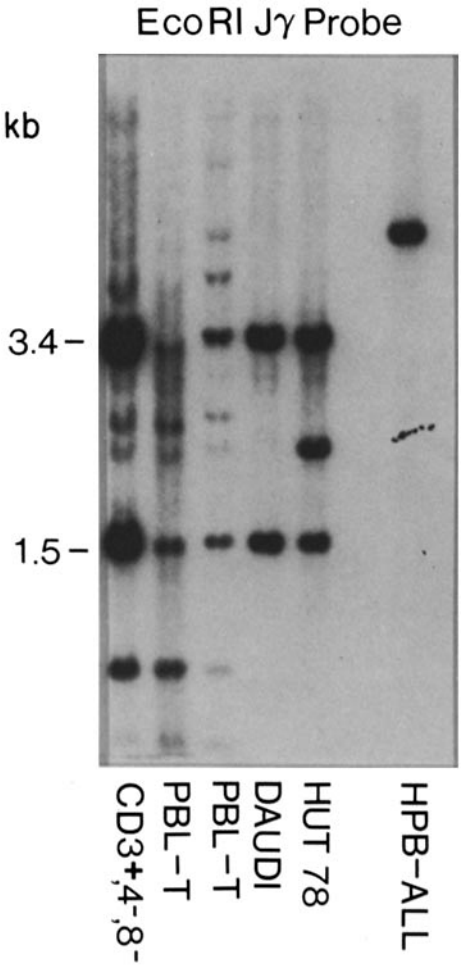

FIGURE 8. Southern blot analysis of γ gene rearrangement in CD3⁺,4⁻,8⁻ T cells. Genomic DNA (10 μg, as determined by spectrophotometric determination) from an IL-2-dependent CD3⁺,4⁻,8⁻ (WT31⁻) cell line, a B cell line (DAUDI), normal PBL/T lymphocytes from two different individuals, HUT 78 T leukemia cells and HPB-ALL T leukemia cells were digested with Eco RI restriction enzyme. Gels were stained with ethidium bromide to verify the integrity of the DNA and to confirm that comparable amounts of DNA were present in all lanes. DNA were analyzed by the Southern blot technique using ^{32}P-labeled Jγ probe.

strated rearrangement of Cβ1 genes, as reflected by extensive loss of the 10-kb fragment. Loss of the 10-kb fragment, without appearance of discrete rearranged fragments, supports the conclusion that CD3⁺,4⁻,8⁻ (WT31⁻) cell lines are polyclonal. Restriction enzyme analysis using Bam HI (to detect Cβ2 gene rearrangements [46]), demonstrated that CD3⁺,4⁻,8⁻ (WT31⁻) cell lines were essentially identical to polyclonal PBL/T cells and did not contain clonal rearrangements (not shown).

Relationship of Normal Peripheral Blood CD⁺,4⁻,8⁻ T Cell Lines to Other CD3⁺,WT31⁻ Cell Lines. PEER T leukemia cells (25), IL-2-dependent CD3⁺,4⁻,8⁻ clones established from cultured thymocytes (27), and IL-2-dependent CD3⁺ T cell lines established from immunodeficiency patients with bare lymphocyte syndrome and ectodermal dysplasia syndrome (26) all failed to react with WT31 mAb. Both the thymic and immunodeficient T cell lines demonstrated two non–disulfide-linked proteins of ~55 and ~40 kD that were coimmunoprecipitated with anti-CD3 mAbs. The PEER cell line expressed an ~55 kD glycoprotein. Although an ~40-kD protein was not visualized by ^{125}I radiolabeling, this does not exclude the possibility that PEER also expresses a second

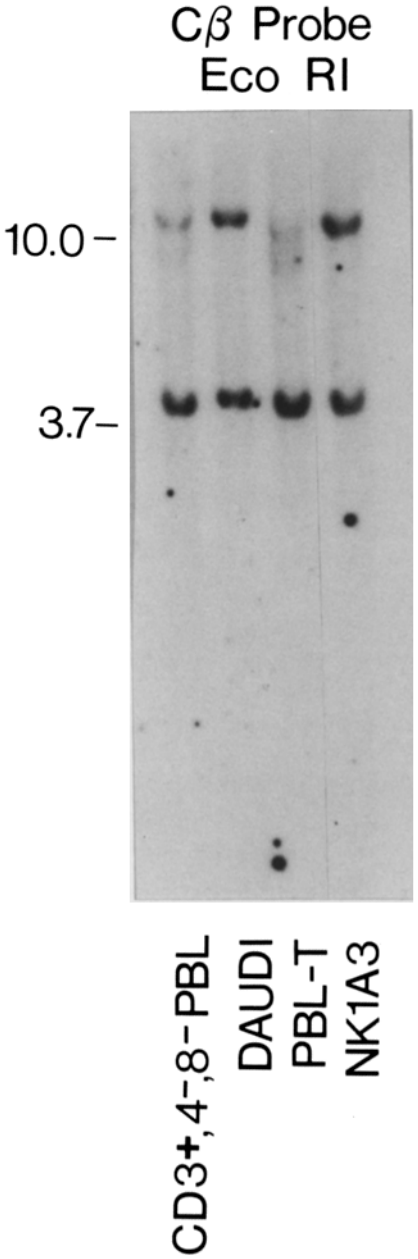

FIGURE 9. Southern blot analysis of β gene rearrangement in $CD3^+,4^-,8^-$ T cells. Genomic DNA (10 μg, as determined by spectrophotometric determination) from an IL-2-dependent $CD3^+,4^-,8^-$ (WT31$^-$) cell line, a B cell line (DAUDI), normal PBL/T lymphocytes, and an IL-2-dependent $CD3^-$ NK cell clone (NK1A3) were digested with Eco RI restriction enzyme. Gels were stained with ethidium bromide to verify the integrity of the DNA and to confirm that comparable amounts of DNA were present in all lanes. DNA were analyzed by the Southern blot technique using ^{32}P-labeled β probe.

non–disulfide-linked subunit. The non–disulfide-linked ~55-kd protein expressed by the immunodeficient T cell lines (26) and PEER (28) reacted with antiserum against γ peptides. Similarly, the ~40- and ~37-kD subunit of the disulfide-linked heterodimer expressed on $CD3^+,4^-,8^-$ cell lines established from normal, adult peripheral blood reacted with anti-γ chain antiserum. In contrast, the ~45-kD subunit of the disulfide-linked heterodimer failed to react with anti-γ serum. Whether or not this ~45-kD subunit of the disulfide-linked heterodimer is related to the ~40-kD subunit of the non–disulfide-linked heterodimer ob-

served on immunodeficient T cell lines is unknown. Brenner and colleagues (26) have proposed that this ~40-kD protein may be encoded by a fourth T cell antigen receptor gene, tentatively designated TCR-δ.

The existence of non–disulfide-linked heterodimers and disulfide-linked heterodimers containing γ chain proteins may reflect preferential use of the two different Cγ genes. Normal peripheral blood $CD3^+,4^-,8^-$ ($WT31^-$) T cells may exclusively transcribe the Cγ1 gene. The Cγ1 gene has a cysteine residue proximal to the transmembrane segment that is potentially available to form interchain disulfide-linked dimers (28, 48). The Cγ2 gene used by PEER (and possibly the thymic and immunodeficient T cell lines) lacks this cysteine residue proximal to the transmembrane region (28, 48). It will be of interest to determine whether differential expression of Cγ1 and Cγ2 genes will correlate with function, stage of differentiation or tissue origin of T lymphocyte expressing γ chain receptors. Preferential expression of Vγ genes in murine fetal thymocytes and mature T lymphocytes has been observed by Garman and colleagues (49).

Like $CD3^+,4^-,8^-$ cell lines established from adult peripheral blood, F6C7, an IL-2-dependent $CD3^+,WT31^-$ T cell clone established from fetal blood, also expresses an ~85-kD disulfide-linked structure associated with the CD3 complex (50). Upon reduction of the ~85-kD structure, only an ~44-kD subunit was visualized, suggesting that this structure may be a homodimer (50). F6C7 cells transcribe apparently functional 1.3-kb β mRNA, as well as 1.6-kb γ mRNA, but not α mRNA (50). There was no indication whether or not this homodimer is a γ gene product. However, if this disulfide-linked antigen receptor is encoded by γ, it is probably using the Cγ1 gene.

At present, there is insufficient data to explain the relationship between the majority of T lymphocytes that express an α/β antigen receptor versus the infrequent subset of T lymphocytes that express a γ/δ receptor in association with the CD3 complex. Moreover, the biological significance of the preferential use of Cγ1 and Cγ2 genes to encode non–disulfide-linked versus disulfide-linked receptors is unclear. These questions are of particular interest in relation to an understanding of T cell ontogeny and differentiation. It has been proposed that T lymphocytes expressing TCR-γ proteins represent a developmental stage of T cell maturation, and that immature thymocytes may express an heterodimeric antigen receptor composed of γ chain and β chain subunits (22, 51). By this scenario, immature TCR-γ/β T lymphocytes would progress to express a TCR-α/β and CD4 and/or CD8 antigens, as a consequence of differentiation, presumably in the thymus. Our present data, as well as the observations of others (26, 27), have clearly demonstrated that the γ chain heterodimers expressed on both peripheral and thymic T lymphocytes do not contain β chain subunits. Moreover, even in the PEER cell line, in which β chain protein is detected in the cytoplasm, no γ/β heterodimers were detected on the plasma membrane (28).

T lymphocytes expressing γ chain antigen receptors are more abundant in adult peripheral blood than in thymus, indicating that cells expressing γ chain antigen receptors do not represent a major stage of thymic development (29). We have suggested that T cells expressing a functional CD3/antigen receptor complex using TCR-γ proteins may represent an independent lineage of T lymphocytes, distinct from the majority of T cells expressing an α/β antigen receptor (29). Additionally, since most T lymphocytes with cell surface TCR-γ

receptors express neither CD8 nor CD4 (antigens that have been implicated in class I and class II MHC interactions, respectively), antigen recognition by these cells may lack restriction by the MHC. The ability to isolate and establish stable in vitro cultures of T cells expressing TCR-γ antigen receptors will provide the opportunity to resolve these questions.

Summary

IL-2-dependent cell lines were established from normal peripheral blood T lymphocytes that express neither CD4 nor CD8 differentiation antigens. $CD3^+,4^-,8^-$ cell lines from 15 different donors failed to react with WT31, an mAb directed against the T cell antigen receptor α/β heterodimer. Anti-Leu-4 mAb was used to isolate the CD3/T cell antigen receptor complex from ^{125}I-labeled $CD3^+,4^-,8^-$ ($WT31^-$) T cells. Using detergent conditions that preserved the CD3/T cell antigen receptor complex, an ~90 kD disulfide-linked heterodimer, composed of ~45- and ~40- (or ~37-) kD subunits, was coimmunoprecipitated with the invariant 20–29-kD CD3 complex. Analysis of these components by nonequilibrium pH gradient electrophoresis indicated that the ~40-kD and ~37-kD subunits were similar, and quite distinct from the more basic ~45-kD subunit. None of these three subunits reacted with an antibody directed against a β chain framework epitope. Heteroantiserum against a T cell receptor γ chain peptide specifically reacted with both the ~37- and ~40-kD CD3-associated proteins, but not with the ~45-kD subunit. $CD3^+,4^-,8^-$ cells failed to transcribe substantial amounts of functional 1.3-kb β or 1.6-kb α mRNA, but produced abundant 1.6-kb γ mRNA. Southern blot analysis revealed that these $CD3^+,4^-,8^-$ cell lines rearranged both γ and β genes, and indicated that the populations were polyclonal. The expression of a CD3-associated disulfide-linked heterodimer on $CD3^+,4^-,8^-$ T cell lines established from normal, adult peripheral blood contrasts with prior reports describing a CD3-associated non–disulfide-linked heterodimer on $CD3^+/WT31^-$ cell lines established from thymus and peripheral blood obtained from patients with immunodeficiency diseases. We propose that this discrepancy may be explained by preferential usage of the two Cγ genes in T lymphocytes.

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